GENERATION OF SPECIFIC ADHESION IN GRAM-NEGATIVE BACTERIA BY MEANS OF ANCHORING IMMUNOGLOBULIN SINGLE DOMAINS ON THEIR SURFACE WITH AUTOTRANSPORTERS

FIELD OF THE INVENTION

The present invention refers to the use of genetic tools which allow the expression of different proteins on the surface of gram-negative bacteria. The present invention particularly refers to the use of autotransporters as carriers for the presentation of recombinant antibodies which specifically bind to known antigens and to thus redirect the bacterial adhesion.

BACKGROUND OF THE INVENTION

The proteins located on the surface of the bacteria, or secreted to the extracellular medium, are necessary for a number of biological phenomenons, such as cell recognition, host cell adhesion and invasion, conjugation, bacteriophage assembly and cell mobility, inter alia (Buttner and Bonas, Trends Mircobiol 2002, 10, 186-192; Cabanes et al., Trends Microbiol 2002, 10, 238-245; Cao and Saier, Microbiology 2001, 147, 3201-3214; Christie, Mol Microbiol 2001, 40, 294-305; Fernández and Berenguer, FEMS Microbiol Rev 2000, 24, 21-44; Finlay and Falkow, Microbiology and Molecular Biology Reviews 1997, 61, 136-169; Giron et al., Mol Microbiol 2002, 44, 361-379; Hueck, Microbiol Mol Biol Rev 1998,, 62, 379-433; Lee and Schneewind, Genes Dev 2001, 15, 1725-1752; Soto and Hultgren, J Bacteriol 1999, 181, 1059-1071).

Protein secretion is particularly complex in gram-negative bacteria, since they must pass through two lipid membranes. To carry out this task, gram-negative bacteria have developed an array of secretion systems classified in five large groups depending on the molecular nature of the transport machinery.

It is also possible to point out the existence of systems specialized in fimbria secretion (chaperone/usher). These systems are mechanistically divided into two large groups: those in which there is no periplasmic intermediary (*sec*-independent), and those with secretion with one stage in the periplasm (*sec*-dependent).

Autotransporters (ATs) were initially disclosed as a family of secreting proteins, all the information necessary for their translocation through the outer membrane (OM) resides in the polypeptide that is exported. The C-terminal region of the ATs acts as a secretion mechanism, translocating the N-terminal domain through the OM without the aid of any other known protein (Henderson et al., Trends Microbiol 2000, 8 (12): 529-532; Henderson et al., Trends Microbiol 1998, 6 (9): 370-8; Holland, Trends Microbiol 1998, 6 (10): 388-9; Pohlner et al., Nature 1987, 325 458-462). The ATs are a widely distributed family among gram-negative bacteria. To date, about 120 members belonging to 20 different genres of α-, β-, γ- and ε-proteobacteria and chlamydias have been identified (Yen et al., Biochim Biophys Acta 2002, 1562 (1-2): 6-31).

Some examples of ATs as virulence factors present in human pathogens are *Neisseria gonorrhoeae, N. meningitidis* and *Haemophilus influenzae* IgA proteases (Pohlner et al., Nature 1987, 325 458-462; Lomholt et al., Mol. Microbiol. 1995, 15 495-506), the polymerization factor of *Shigella flexnerii* IcsA actin (Suzuki et al., J. Biol. Chem. 1995, 270 30874-30880), AIDA-I adhesin of pathogenic *Escherichia coli* strains (Benz and Schmidt, Mol Microbiol 1992, 6, 1539-1546; Suhr et al., Mol. Microbiol. 1996, 22, 31-42), the factor of resistance to the *Bordetella pertussis* BrkA complement (Fernández and Weiss, Infect. Immun. 1994, 62 4727-4738), and the *Helicobacter pylori* VacA cytotoxin (Schmitt and Haas, Mol. Microbiol. 1994, 12 307-319).

All the ATs have the same modular structure (see Figure 1). From the N-terminal end: **I**, a signal peptide directing the translocation of the pre-protein towards the periplasm through the inner membrane (IM) (Henderson et al., Trends Microbiol 1998, 6, 370-378; Pohlner et al., Nature, 1987, 325, 458-462; Sijbrandi et al., J Biol Chem 2002, 3, 3); **II**, a variably-sized passenger domain (from 0 to more than 250 kDa), which is translocated through the OM and is responsible for the biological extracellular role; **III**, a transporter domain (β) located in the C-terminal end (sometimes referred herein as C-terminal β-domain or C-terminal domain or transporter), conserved among the members of the family, and that directs the secretion through the OM of the passenger domain (Pohlner et al., Nature, 1987, 325, 458-462; Klauser et al., J. Mol. Biol. 1993, 234 579-593; Maurer et al., J Bacteriol 1999, 181 (22): 7014-20).

The passenger domain can be processed and released to the medium (*N. gonorrhoeae* IgA protease), it can be processed and remain associated to the OM (*E. coli* Ag43 adhesin) or it can go unprocessed and remain exposed towards the medium (*H. influenzae* Hia adhesin).

Several members of the family of the ATs have been used for the exposure of different polypeptides fused to the β domain thereof, such as rat metallothionein for example (Valls et al., Nat. Biotechnol. 2000, 18; 661-5), cholera toxin subunit B (Maurer et al., J. Bacteriol. 1997, 794-804), β-lactamase (Lattemann et al., J. Bacteriol. 2000, 182: 3726-33), bovine adrenodoxin (Jose et al., J. Biotechnol. 2002, 95: 257-68), carboxylesterase EstA from *Burkholderia gladioli* (J. Mol. Catal. B: Enzymatic 2002, 18: 89-97), the fimbrial protein FimH (Kjaergaard et al., J. Bacteriol. 2002, 184: 4197-204), inter alia.

The work aimed at the exposure of polypeptides on the bacterial surface by means of the use of different members of

the family of ATs indicates the need to prevent systems with disulfide bonds (Jose, J. et al., 1996, 178 (107-110); (Klauser, T. et al., 1992, 11 (2327-2335)). This fact is also clearly shown in the work of Veiga et al. (Mol. Microbiol. 1999, 33(6): 1232-1243), which discloses the secretion and translocation as a passenger domain of a single chain Fv fragment (which has two intramolecular disulfide bonds stabilizing their tertiary structure and which are required for scFvs to be functional), this translocation is achieved with a low efficiency rate, having problems of proteolysis of the fusion, in addition to problems of toxicity for the bacteria.

The miniantibodies or recombinant antibodies are fragments derived from the antibodies built by recombinant DNA technology, and in spite of their smaller size, they conserve the antigen binding capacity given that they maintain the variable domains (V) of an immunoglobulin (Ig), where the antigen binding areas are located.

One type of miniantibodies are those referred to as scFv (Plückthun, et al., Antibody Engineering: A Practical Approach 1996), 203-252). These scFv are chimeric proteins of about 30 kDa resulting from the fusion in the same polypeptide chain of the variable domains (V_H and V_L) of the heavy (H) and light (L) chains of an antibody through a flexible peptide (usually 20 amino acid repetitions of glycine and serine) allowing the quaternary interactions between both Ig domains to be conserved. The binding capacity of these scFv resides in the surface generated by the interaction of the two variable domains. These scFv have two intramolecular disulfide bonds stabilizing their tertiary structure and required for the scFv to be functional.

Other miniantibodies successfully expressed in *E. coli* are those called V_{HH} (Nguyen et al., Adv Immunol 2001, 79 261-296; Nguyen et al., Embo J 2000, 19 (5): 921-30; Sheriff and Constantine, Nat Struct Biol 1996, 3 (9): 733-6). In camelidae

species (camels, dromedaries, llamas, etc.), part of their antibodies is light-chain deficient (Hamers-Casterman et al., Nature 1993, 363 (6428): 446-8; Muyldermans et al., Protein Eng 1994, 7 (9): 1129-35), the recognition area of the antigens being constituted only of the V_H domain (Desmyter et al., J Biol Chem 2001, 276 (28): 26285-90; Desmyter et al., J Biol Chem 2002, 277 (26): 23645-50; Desmyter et al., Nat Struct Biol 1996, 3 (9): 803-11). This V_H domain differs from the V_H domains of other antibodies in the sense that its antigen recognition areas are formed by larger loops. Thus, the camelidae antibody V_H domains are called V_{HH}. This about 15 kDa domain (V_{HH}) can be expressed in the *E. coli* periplasm maintaining its antigen binding capacity. The V_{HH} are more stable molecules than the scFv, and unlike the latter, they rarely aggregate (J Immunol. Methods 1999, 231: 25-38).

Just as V_L and V_H domains in mice, V_{HH} domains have an intramolecular S-S bond stabilizing their tertiary structure and necessary for their proper folding (Desmyter et al., J Biol Chem 2002, 277 (26): 23645-50; Desmyter et al., Nat Struct Biol 1996, 3 (9): 803-11).

Veiga et al. (Mol Microbiol 1999, 33: 1232-1243) disclosed the secretion of an scFv miniantibody (which has two intramolecular disulfide bonds stabilizing its tertiary structure) as a passenger fused to the C-terminal domain of *N. gonorrhoeae* IgA protease (C-IgAP) with an efficiency of about 20%, further having problems of proteolysis of the fusion and of toxicity for the bacteria.

SUMMARY OF THE INVENTION

According to the present invention, the anchoring and expression of recombinant antibodies with a single immunoglobulin domain (single-domain) on the surface of the outer membrane (OM) of a bacteria, i.e., on the external surface of the bacterial OM, has been unexpectedly achieved by using a transporter domain of an autotransporter (AT) with a

notably higher efficiency than that expected, and decreasing the problems both of proteolysis of the fusion and toxicity for the bacteria, in spite of the fact that said single-domain antibodies have disulfide bonds in their tertiary structure.

Therefore, in an aspect, the invention relates to a gene construction comprising (i) a nucleotide sequence encoding a signal peptide, (ii) a nucleotide sequence encoding a single-domain recombinant antibody; and (iii) a nucleotide sequence encoding the C-terminal domain of an AT; wherein the 3′ end of said sequence (i) is linked to the 5′ end of said sequence (ii) and the 3′ end of said sequence (ii) is linked to the 5′ end of said sequence (iii).

In other aspect, the invention relates to an expression vector comprising said gene construction operatively linked to a transcription control sequence. In an embodiment said transcription control sequence is functional in bacteria.

An expression vector for single-domain recombinant antibodies on the surface of the bacterial OM, characterized in that the expressed antibody is secreted by a transporter domain of an AT, constitutes an additional aspect of this invention.

In other aspect, the invention relates to gram-negative bacteria comprising said gene construction or said expression vector.

In other aspect, the invention relates to a hybrid protein obtainable by the expression of nucleic acid sequence contained in said gene construction or in said expression vector.

In other aspect, the invention relates to a method of anchoring and expressing a single-domain recombinant antibody on the surface of the OM of a bacteria which comprises culturing a bacteria containing said gene construction or said expression vector under conditions which allow for the production of said single-domain recombinant antibody, its

anchoring and expression on the surface of the OM of said bacteria in the form of a hybrid protein.

In other aspect, the invention relates to a method for the specific adhesion of a bacteria to an antigen, wherein said bacteria has a single-domain recombinant antibody capable of recognizing (binding to) said antigen anchored on the surface of its OM.

In other aspect, the invention relates to a method for producing a single-domain recombinant antibody anchored on the surface of the OM of a bacteria.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a representation of the modular organization of the ATs. The N and C terminal ends are indicated, as well as the signal peptide (sp), the translocated passenger domain and the β domain.

Figure 2 shows a representation of the basic structure of a expression vector of the fusions between immunoglobulin V domains and transporter domains. The encoding DNA of the V domain is between encoding sequences of a signal peptide (SP) and the autotransporter domain (AT). The V domain is ideally flanked by two single restriction sites (R1, R2) and the V-AT fusion contains an epitope (E) for detection thereof by means of a specific antibody. The vector further contains the following elements: a replication origin (ori), a marker gene (M) for its selection, and promoter sequences (P) for the transcription of the V-AT fusion.

Figure 3 shows: (A) A representation of the modular organization of $V_{amy}\beta$, wherein the N and C terminal ends are indicated, as well as the signal peptide (sp), the translocated passenger domain V_{amy} (represented as V_{HH} in the figure) and the C-terminal β-domain of *N. gonorrhoeae* IgA protease (C-IgAP). (B) A representation of the modular organization of $V_{LMB10}\beta$, wherein the N and C terminal ends are

indicated, as well as the signal peptide (sp), the translocated passenger domain V_{LMB10} (represented as V_L* in the figure) and the C-terminal β-domain of *N. gonorrhoeae* IgA protease (C-IgAP). Both proteins include an epitope (E-TAG) for its detection by a specific antibody.

Figure 4 shows: (A) Immunodetection by Western-blot using the anti-E-tag monoclonal antibody (mAb) of the different hybrid proteins ($V_{amy}\beta$ and FvHβ) from total *E. coli* UT5600 cell extracts. In the case of $V_{amy}\beta$, a single band corresponding to 66 kDa is observed, whereas in the case of FvHβ, several bands corresponding to the proteolysis of the fusion are observed. (B) Exposure of the $V_{amy}\beta$ and FvHβ hybrid proteins on the surface of *E. coli* UT5600. When (+) is indicated, the cells were permeabilized with 10 mM EDTA (ethylendiaminetetraacetic acid), or they were incubated with trypsin (1 µm/ml) added externally. The presence of the different hybrid proteins in the crude extracts was determined by Western-blot with the anti-E-tag mAb. The amount of OmpA in the same extracts (determined using a polyclonal rabbit anti-OmpA serum) is shown as a loading control. To detect the about 24 kDa fragment (T-OmpA) corresponding to the tryptic degradation of the periplasmic domain of OmpA, the photographic films were overexposed.

Figure 5 shows a functionality/folding assay of the passenger domains of the different constructs derived from C-IgAP exposed towards the extracellular medium. ELISA plates were coated with α-amylase (Amy) and with bovine serum albumin (BSA) as an antigen control. The *E. coli* UT5600 cells which expressed these hybrid proteins were added on the ELISA plates and the binding of the passengers exposed on the surface of these cells was detected with the anti-E-tag mAb linked to peroxidase (POD). The *E. coli* cells which expressed the HEβ protein were used as a negative control.

Figure 6 shows the exposure of the $V_{LMB10}\beta$ hybrid on the surface of *E. coli* UT5600. When (+) is indicated, the cells were permeabilized with 10 mM EDTA, or they were incubated with trypsin (1 µg/ml) added externally. The presence of the different hybrid proteins in the crude extracts was measured by Western-blot with the anti-E-tag mAb. The amount of OmpA in the same extracts (measured using a polyclonal rabbit anti-OmpA serum) is shown as a loading control. An exposure of over 90% of the fusion protein (65 kDa band) is observed, there also being a single band of proteolytic degradation of about 55 kDa.

Figure 7 shows: (A) The modular organization of $2V_{amy}\beta$ and $3V_{amy}\beta$. (B) The exposure of the $2V_{amy}\beta$ and $3V_{amy}\beta$ hybrid proteins on the surface of *E. coli* UT5600. When (+) is indicated, the cells are incubated with trypsin (1 µg/ml) added externally. The presence of the different hybrid proteins in the crude extracts was measured by Western-blot with the anti-E-tag mAb. The amount of OmpA in the same extracts (measured using a polyclonal rabbit anti-OmpA serum) is shown as a loading control.

Figure 8 shows the peptidoglycan (PG) accessibility assay in which the control (pAKnot), $V_{amy}\beta$, $2V_{amy}\beta$, $3V_{amy}\beta$ and $V_{LMB10}\beta$ constructs were included. In all of them, only one PG accessibility level occurs at about 5% maximum and identical to that observed in the control strain which does not express any hybrid protein.

Figure 9 shows the specific adhesion of *E. coli* cells to a surface containing an amylase antigen (Amy) or control antigen (BSA). The binding of the *E. coli* cells to the plates was detected with a polyclonal anti-*E. coli* serum generated in mice. The *E. coli* cells which expressed the HEβ protein were used as a negative control.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates, in general, to the anchoring and expression of single-domain recombinant antibodies on the bacterial surface, secreted by a transporter domain of an AT. Thus, the invention provides an expression vector for single-domain recombinant antibodies on the surface of the bacterial OM, characterized in that the expressed antibody is secreted by a transporter domain of an AT.

Therefore, in an aspect, the invention relates to a gene construction, hereinafter referred to as the gene construction of the invention, comprising:

a) a first nucleic acid sequence containing the nucleotide sequence encoding a signal peptide;

b) a second nucleic acid sequence containing the nucleotide sequence encoding a single-domain recombinant antibody; and

c) a third nucleic acid sequence containing the nucleotide sequence encoding the C-terminal domain of an AT;

wherein the 3′ end of said first nucleic acid sequence is linked to the 5′ end of said second nucleic acid sequence and the 3′ end of said second nucleic acid sequence is linked to the 5′ end of said third nucleic acid sequence.

The first nucleic acid sequence comprises the nucleotide sequence encoding a signal peptide. A signal peptide is a peptide sequence, usually present in the N-terminal end of may secretory proteins (i.e., which are secreted or exported from the place they are produced) or membrane proteins, involved in the passage of the protein across the cell membrane (in bacteria). Practically any signal peptide may be used in the instant invention. Illustrative, non limitative, examples of said signal peptide directing the passage of the hybrid protein provided by the instant invention towards the periplasm include the signal peptide of PelB (Keen, N.T. et al., J Bacteriol 1986, 168 (2) 595-606), the signal peptide of OmpA (Movva, N.R. et al., J Biol Chem 1980, 255 (1) 27-9), the

signal peptide of protein 3 of bacteriophage M13 (Parmley, S.F. et al., Adv Exp Med Biol 1989, 251 (215-8)), the signal peptide of the maltose binding protein (MBP) (Bedouelle, H. et al., Eur J Biochem 1988, 171 (3) 541-9), or any other signal peptide of those normally used with AT systems. In a particular embodiment, said signal peptide is the signal peptide of PelB.

The second nucleic acid sequence comprises the nucleotide sequence encoding a single-domain recombinant antibody. Within the framework of the present invention, a "single-domain recombinant antibody" refers to an immunoglobulin-type domain with independent binding and recognition capacity, such as a natural or modified heavy chain variable domain (V_H) of an antibody, a natural or modified light chain variable domain (V_L) of an antibody, a natural or modified recombinant camelide antibody (V_{HH}), a humanized recombinant camelide antibody, a recombinant antibody of a non-camelide animal engineered in order to make it capable of interacting in the form of a single-domain with its antigen (i.e., "camelized"), an IgNAR single-domain antibody of cartilaginous fish, etc. The gene construction of the invention may contain a combination of nucleotide sequences encoding single-domain recombinant antibodies. The gene construction of the invention may contain a nucleotide sequence encoding one single-domain recombinant antibody or the nucleotide sequences encoding two or more, equal or different. In the last case, said single-domain recombinant antibodies may be, optionally, separated from each other by some spacers (i.e., peptide sequences which allow for separating said single-domain recombinant antibodies each other, which will be described later in a detailed manner). In a particular embodiment, the gene construction of the invention comprises a nucleotide sequence encoding just one single-domain recombinant antibody. In another particular embodiment, the gene construction of the invention comprises

the nucleotide sequences encoding two or more, for example, three, equal or different single-domain recombinant antibodies. Said single-domain recombinant antibody or antibodies constitute, therefore, the passenger domain or domains to be secreted by the AT system present in the gene construction of the invention or in the expression vectors provided by this invention.

As used herein, the term "natural", applied to a product, refers to the product as found in nature, without having been subjected to human manipulation, whereas the term "modified" refers to any change carried out by the human being on a natural product, e.g., changes introduced in natural products in order to modify or improve their stability and/or solubility properties. Also, the term "non-camelide animal" refers to an animal capable of generating immunoglobulins which does not pertain to the camelidae family [family of mammals of the artiodactyls order, tilopodes suborder]. Therefore, the term "single-domain recombinant antibody", as used herein, includes both the antigen recognition regions of antibodies which are naturally single-domain (e.g., V_{HH} and IgNAR), and antigen recognition regions of antibodies which, by genetic engineering, have been altered so that they are able to interact with the antigen on their own and improve their properties of stability and/or solubility.

Although practically any single-domain antibody capable of being recombinantly expressed may be used in the instant invention, in a particular embodiment, with illustrative, non limitative, purposes, of the invention, a V_{HH} recognizing α-amylase (V_{amy}) as representative of a natural single-domain recombinant antibody was used (Example 1). Said V_{amy} contains a single Ig domain, its size being the half (about 15 kDa) of the size corresponding to an scFv (about 30 kDa). As representative of a genetically engineered single-domain recombinant antibody so that it would be able to interact with the antigen on its own and improve its stability and

solubility properties, a V_L of human origin (MB10 clone) against the B7.1 cell surface marker (van den Beucken et al., J. Mol. Biol. (2001) 310, 591-601) was used (Example 2).

The third nucleic acid sequence comprises the nucleotide sequence encoding the C-terminal domain of an AT. The C-terminal domain (transporter) of an AT acts as a secretion machinery translocating the N-terminal end through the OM. Practically, for carrying out the invention it may be used the C-terminal domain of any AT capable of translocating the passenger domain (single-domain recombinant antibody or antibodies) to which it is bound, across the OM of a bacteria, its anchoring and exposure on the external surface of the OM of said bacteria. In a particular embodiment, said third nucleic acid sequence comprises the nucleotide sequence encoding the C-terminal domain of an AT of a gram-negative bacteria. Illustrative, non limitative, examples of said ATs include IgA proteases of *Neisseria spp.* or *Hemophilus spp.*, for example, *N. gonorrhoeae*, *N. meningitides*, *H. influenzae* IgA proteases (Pohlner et al., Nature 1987, 325 458-462; Lomholt et al., Mol. Microbiol. 1995, 15 495-506), the polymerization factor of *Shigella flexnerii* IcsA actin (Suzuki et al., J. Biol. Chem. 1995, 270 30874-30880), AIDA-I adhesin of pathogenic *Escherichia coli* strains (Benz and Schmidt, Mol Microbiol 1992, 6, 1539-1546; Suhr et al., Mol. Microbiol. 1996, 22, 31-42), the factor of resistance to the *Bordetella pertussis* BrkA complement (Fernández and Weiss, Infect. Immun. 1994, 62 4727-4738), the *Helicobacter pylori* VacA cytotoxin (Schmitt and Haas, Mol. Microbiol. 1994, 12 307-319), etc. In a specific embodiment, said third nucleic acid sequence comprises the nucleotide sequence encoding the C-terminal β-domain of *N. gonorrhoeae* IgA protease. Information concerning sequences of AT is available to the public and may be obtained, for example, from http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF03797.

The expression of the nucleic acid sequence contained in the gene construction of the invention in a suitable expression system results in a hybrid protein (fusion protein) which comprises a domain (A) comprising the amino acid sequence of, at least, a single-domain recombinant antibody, and a domain (B) comprising the amino acid sequence of the C-terminal domain of an AT.

Generally, said domain (A) is not directly fused to said domain (B) since it may be advantageous to introduce between them a spacer (peptide sequence which allows for separating said domains (A) and (B) from each other). Therefore, if desired, the gene construction of the invention may contain, in addition, a fourth nucleic acid sequence encoding a spacer placed between said second and third nucleic acid sequences, wherein the 5′ end of said fourth nucleic acid sequence is linked to the 3′ end of said second nucleic acid sequence and the 3′ end of said fourth nucleic acid sequence is linked to the 5′ end of said third nucleic acid sequence. Thus, the nucleotide sequence encoding the single-domain recombinant antibody or antibodies is joined to the nucleotide sequence encoding the C-terminal domain of an AT by means of a nucleotide sequence encoding a spacer. Advantageously, said spacer is a peptide sequence having structural flexibility (i.e., flexible). Practically any peptide sequence having structural flexibility may be used. Said flexible peptide sequence may comprise, for example, multimers or repetitions of amino acid residues, such as, alanine (A), glycine (G), etc., the peptide sequence -AAAAGA- or any other suitable non repetitive sequence of amino acid residues such as, for example, the non repetitive spacer of sequence -TPSHNSHQVPSAGGPTANSG- (one-letter code of amino acids), etc., or the hinge region of an antibody.

When the hybrid protein provided by the instant invention as a result of the expression of the nucleic acid sequence contained in the gene construction of the invention

comprises two or more single-domain recombinant antibodies, said antibodies may be, optionally, separated from each other by spacers of the previously defined type, so that said single-domain recombinant antibodies are not directly fused between them but through said spacers. Therefore, if desired, the gene construction of the invention may further contain a nucleic acid sequence encoding a spacer placed between two nucleic acid sequences encoding said single-domain recombinant antibodies. Thus, the nucleotide sequences encoding the single-domain recombinant antibodies are joined to each other by a nucleotide sequence encoding a spacer. The same spacer used for separating the domains (A) and (B), previously defined, may be used for separating the single-domain recombinant antibodies from each other. In a particular embodiment, the spacer used is the non repetitive spacer of sequence -TPSHNSHQVPSAGGPTANSG- (one-letter code of amino acids) (Example 1, A48 constructions and V_{amy} repetitions).

In order to make easier the detection of the hybrid protein provided by the instant invention as a result of the expression of the nucleic acid sequence contained in the gene construction of the invention, said gene construction may contain, if desired, a nucleic acid sequence encoding a peptide sequence susceptible of being used for detection or recognition purposes of the hybrid protein of the invention. Therefore, in a particular embodiment, the gene construction of the invention comprises, if desired, a fifth nucleic acid sequence encoding a peptide sequence for detection purposes. Practically any peptide sequence which allows for detecting the hybrid protein of the invention may be used, for example, a peptide sequence susceptible of being recognized by an antibody (e.g., a monoclonal antibody) which can be used for recognizing or detecting the hybrid protein of the invention by conventional analytical methods (e.g., immunoaffinity chromatography, etc.), for example, a poly-hystidine sequence, the sequences of epitopes E, HA, FLAG, c-myc, etc. [Using

Antibodies: A laboratory manual. Ed Harlow and David Lane (1999). Cold Spring Harbor Laboratory Press. New Cork. Capítulo: Tagging proteins. pp. 347-377] and, in general, any other sequence capable of being recognized by an antibody. In a particular embodiment, said peptide sequence susceptible of being used with detection purposes comprises the sequence of epitope E (E-tag).

The above mentioned fifth nucleic acid sequence is placed, advantageously, between said second and third nucleic acid sequences, wherein the 5′ end of said fifth nucleic acid sequence is linked to the 3′ end of said second nucleic acid sequence and the 3′ end of said fifth nucleic acid sequence is linked to the 5′ end of said third nucleic acid sequence. Nevertheless, said fifth nucleic acid sequence could be placed in any other position provided that the presence of said sequence guarantees the detection of the hybrid protein of the invention and does not affect the functionality of said hybrid protein.

The gene construction of the invention may be obtained by using techniques broadly known by the skilled person in the art [Sambrook et al., "Molecular cloning, a Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press, N.Y., 1989 Vol 1-3]. Said gene construction of the invention may be used for the construction of vectors, such as expression vectors, specially useful for producing in gram-negative bacteria hybrid proteins comprising, at least, a single-domain recombinant antibody and the C-terminal domain of an AT.

Therefore, in other aspect, the invention relates to a vector, hereinafter referred to as vector of the invention, which comprises the gene construction of the invention. In a particular embodiment, the vector of the invention is an expression vector which comprises a gene construction of the invention operatively linked to a transcription control sequence. Consequently, the invention provides an expression vector for single-domain recombinant antibodies on the surface

of the bacterial OM, characterized in that the expressed antibody is secreted by a transporter domain of an AT.

As used herein, "transcription control sequence" refers to a sequence which controls and regulates the transcription of the coding sequences contained in the gene construction of the invention. Said transcription control sequence comprises a promoter, a sequence encoding transcriptional regulators, a ribosome binding sequence (RBS) and/or a transcription terminator sequence. In a particular embodiment, said transcription control sequence is functional in bacteria, e.g., in gram-negative bacteria, and comprises an inducible or constitutive, transcription promoter functional in bacteria. Illustrative, non limitative, examples of inducible promoters include promoter pTetA which is induced by anhydrotetracycline (Skerra A., Gene 1994, 151 131-135); promoter pBAD which is induced by arabinose (Guzman L.M. et al., J Bacteriol 1995, 177(14) 4121-4130), the promoter of *Escherichia coli lac* operon (pLac) or its derivative promoter pTac, both induced by IPTG, lactose and other lactose analogues (Miller, The Operon 1978), promoter Pm which is induced by 3-methyl-benzoate and other benzoate analogues (Ramos, J.L. et al., Annu Rev Microbiol 1997,51 (3) 341-73), etc. Illustrative, non limitative, examples of constitutive promoters include the *E. coli* β-lactamase promoter (Klotsky, R.A. et al., Gene 1987, 55 (1) 141-6). In a particular embodiment, the induction of the expression of the expression vectors provided by this invention is under the control of promoter pLac and said induction may be obtained by adding IPTG, lactose or other lactose analog to the culture medium.

If desired, the vector of the invention may comprise, further, a marker, such as, for example, a gene which encodes a motif or phenotype which permits the selection of the host cell transformed with said vector. Illustrative, non limitative examples of said markers which could be present in the vector of the invention, such as in an expression vector

provided by this invention, include genes of antibiotics resistance, e.g., genes of resistance to amplicillin, tetracycline, kanamycin, chloramphenicol, spectinomicin, etc.

The vector of the invention, once introduced into the host cell, may be integrated (or not) into the genome of said cell.

The vector of the invention may be obtained by using conventional techniques known by the skilled person in the art [Sambrook et al., "Molecular cloning, a Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press, N.Y., 1989 Vol 1-3].

In a particular embodiment, the invention provides an expression vector for gram-negative bacteria which allows for producing hybrid proteins, said hybrid proteins comprising, at least, a single-domain recombinant antibody and the C-terminal domain of an AT.

According to another preferred embodiment of the present invention, an expression vector for gram-negative bacteria is provided which allows for the production of hybrid proteins comprising, at least, a single-domain recombinant antibody and the C-terminal β-domain of *N. gonorrhoeae* IgA protease.

According to another particular embodiment of the present invention, the structure of the expression vectors derived from the *N. gonorrhoeae* IgA protease comprises, starting from the N-terminal end, an inducible or constitutive bacterial transcription promoter, the sequence encoding a signal peptide (which is the one that directs the passage of the hybrid proteins towards the periplasm), the sequence encoding, at least, a single-domain recombinant antibody (passenger domain) and the sequence encoding the C-terminal β-domain of *N. gonorrhoeae* IgA protease (which directs the secretion of the passenger domain towards the extracellular medium). Figure 2 depicts the structure of these vectors. Examples 1 and 2 describe the construction of some expression vectors derived from *N. gonorrhoeae* IgA protease comprising 1, 2 or 3 times the sequence encoding V_{amy} (V_{HH} which recognizes

α-amylase) (Example 1) and the sequence encoding $V_{LMB10}\beta$ (V_L of human origin (MB10 clone) against the B7.1 cell surface marker) (Example 2), together with the encoding sequence of the C-terminal β-domain of *N. gonorrhoeae* IgA protease (C-IgAp).

In other aspect, the invention relates to a bacteria, in particular, a gram-negative bacteria, comprising a gene construction of the invention or an expression vector of the invention, hereinafter referred to as bacteria of invention. Practically any gram-negative bacteria, for example, a *Escherichia spp.* strain (e.g., *E. coli*), etc., a *Salmonella spp.* strain (e.g., *S. tiphymurium*, etc.) and a *Pseudomonas spp.* strain (*P. aeruginosa*, *P. putida*, etc.), may be transformed with a gene construction of the invention or with a vector of the invention such as an expression vector provided by this invention. To that end, the promoter, regulatory and marker signals, as well as the replication origin will be optimized for each bacteria. In a particular embodiment, said gram-negative bacteria is a *E. coli* strain.

The gene construction of the invention as well as the expression vector of the invention may be used for transforming bacteria, in particular, gram-negative bacteria, thus generating transforming bacteria which carry, anchored on the external surface of its OM, at least, a single-domain recombinant antibody, in the form of a hybrid protein fused to the C-terminal domain of an AT; said bacteria being useful, thus, as systems for the presentation of antibodies, and may be used in a number of applications, including, among other:

- applications related to the bacterial adhesion for, for example, chelating metals, neutralizing pathogens (e.g., viral, etc.), specific adhesion to certain plants, bacterial aggregation, etc.;
- applications related to diagnostic purposes, in which case the single-domain recombinant antibody or antibodies present on the external surface of the OM of said bacteria will be an antibody capable of

recognizing, or binding to, an antigen, such as a microbial antigen, i.e., an antigen of a microorganism including, without restricting to, viruses, bacteria, fungi and infectious parasites; a tumor antigen, i.e., a compound associated with a tumor or cancer ("tumor marker"), e.g., Her2 (breast cancer); GD2 (neuroblastome); EGF-R (malignant glioblastome); CEA (medular tyroid cancer); CD52 (leukemia); gp100 protein of human melanome; melan-A/MART-1 protein of human melanome; tyrosinase; NA17-A nt protein; MAGE-3 protein; p53 protein; HPV16E7 protein; etc., an auto-antigen, i.e., a protein encoded by the DNA of a subject and products generated by proteins or by RNA encoded by the DNA of a subject (WO 02/56905); and

- applications related to the selection of antibodies from antibodies collections by "bacterial display".

In other aspect, the invention relates to a hybrid protein, hereinafter referred to as hybrid protein of the invention, obtainable by the expression of the nucleic acid sequence contained in a gene construction according of the invention or in an expression vector provided by this invention.

In an embodiment, the hybrid protein of the invention comprises a domain (A) which comprises the amino acid sequence of, at least, a single-domain recombinant antibody, and a domain (B) comprising the amino acid sequence of the C-terminal domain of an AT. In a particular embodiment, said domain (B) comprises the amino acid sequence of the C-terminal β-domain of *N. gonorrhoeae* IgA protease.

The hybrid protein of the invention may also comprise a spacer between said domains (A) and (B) and/or a peptide sequence susceptible of being used for detection purposes, such as a peptide sequence susceptible of being recognized by

an antibody, such as a poly-hystidine sequence, the sequences of epitopes E, HA, FLAG, c-myc, etc.

In a particular embodiment, hybrid proteins anchored to the external surface of the OM of *E. coli* comprising 1, 2 or 3 V_{amy} domains fused to the C-IgAp domain, referred to as $V_{amy}\beta$, $V_{amy}2\beta$ and $V_{amy}3\beta$ respectively (Figures 3A and 7A) (Example 1), have been obtained. In another particular embodiment, a hybrid protein anchored to the external surface of the OM of *E. coli* comprising the V_{LMB10} domain fused to the C-IgAp domain, hereinafter referred to as $V_{LMB10}\beta$ (Figure 3B) (Example 2). In all the cases, the signal peptide of PelB directs the secretion of said hybrid proteins through the IM to the periplasmic space.

In other aspect, a method of anchoring and expressing a single-domain recombinant antibody on the surface of the OM of a bacteria which comprises culturing a bacteria of the invention under conditions which allow for the production of said single-domain recombinant antibody, its anchoring and expression on the surface of the OM of said bacteria (i.e., in the external surface of said bacterial OM) in the form of a hybrid protein fused to the C-terminal domain of an AT, such as the C-terminal domain of a gram-negative bacteria, e.g., the C-terminal β-domain of *N. gonorrhoeae* IgA protease. In a particular embodiment, said bacteria is a gram-negative bacteria provided by this invention, such as a *Escherichia spp.* strain (e.g., *E. coli*), etc., a *Salmonella spp.* strain (e.g., *S. tiphymurium*, etc.), a *Pseudomonas spp.* strain (*P. aeruginosa*, *P. putida*, etc.), etc. Conditions for optimizing the culture of the bacteria of the invention will depend on the bacteria used.

In other aspect, the invention relates to a method for the specific adhesion of a bacteria to an antigen which comprises the steps of:

a) transforming a bacteria with a gene construction of the invention or with an expression vector, said gene construction or expression vector comprising the nucleotide sequence encoding a single-domain recombinant antibody capable of recognizing said antigen;

b) culturing said transformed bacteria under conditions which allow for the production of said single-domain recombinant antibody, its anchoring and expression on the surface of the OM of said bacteria; and

c) bringing together the transformed bacteria and cultured from step b) with said antigen.

When culturing and growing the transformed bacteria under conditions which allow for the production of said single-domain recombinant antibody [step b)], due to the particulars of the gene construction or vector used, takes place its anchoring and expression on the surface of the OM of said bacteria (i.e., on the external surface of said bacterial OM) of said single-domain recombinant antibody in the form of a hybrid protein fused to the C-terminal domain of an AT, such as the C-terminal domain of a gram-negative bacteria, e.g., the C-terminal β-domain of *N. gonorrhoeae* IgA protease. In a particular embodiment, said bacteria is a gram-negative bacteria provided by this invention, such as a *Escherichia spp.* strain (e.g., *E. coli*), etc., a *Salmonella spp.* strain (e.g., *S. tiphymurium*, etc.), a *Pseudomonas spp.* strain (*P. aeruginosa*, *P. putida*, etc.), etc. Conditions for optimizing the culture of the bacteria of the invention will depend on the bacteria used.

In other aspect, the invention relates to a method for producing a single-domain recombinant antibody anchored on the surface of the OM of a bacteria which comprises culturing a bacteria of the invention under conditions which allow for the production of said single-domain recombinant antibody, its anchoring and expression on the surface of the OM of said bacteria (i.e., in the external surface of said bacterial OM)

in the form of a hybrid protein fused to the C-terminal domain of an AT, such as the C-terminal domain of a gram-negative bacteria, e.g., the C-terminal β-domain of *N. gonorrhoeae* IgA protease. In a particular embodiment, said bacteria is a gram-negative bacteria provided by this invention, such as a *Escherichia spp.* strain (e.g., *E. coli*), etc., a *Salmonella spp.* strain (e.g., *S. tiphymurium*, etc.), a *Pseudomonas spp.* strain (*P. aeruginosa*, *P. putida*, etc.), etc. Conditions for optimizing the culture of the bacteria of the invention will depend on the bacteria used.

The present invention is illustrated by means of the following, non-limiting, examples.

EXAMPLE 1

Construction of hybrid proteins made up of the V_{HH} domain fused to C-IgAP

Hybrid proteins anchored to the external surface of the OM of *E. coli* comprising 1, 2 or 3 V_{amy} domains fused to the C-IgAp domain, hereinafter referred to as $V_{amy}\beta$, $V_{amy}2\beta$ and $V_{amy}3\beta$ respectively (Figures 3A and 7A), have been obtained. The signal peptide of PelB drives the secretion of said hybrid proteins through the IM to the periplasmic space.

1.1 Construction of the C-IgAP and V_{amy} hybrid protein ($V_{amy}\beta$)

An approximately 0.4 kb DNA fragment encoding a V_{HH} domain recognizing α-amylase (V_{amy}) was amplified by PCR using the phagemid A100R3A2 (Dyax Co.) as a template and the oligonucleotides VHAA1 (SEQ ID NO: 3) and GEN III-Rev (SEQ ID NO: 4) as primers. The amplified DNA product was subsequently digested with SfiI and NotI and cloned into an approximately 5.2 kb fragment derived from the digestion of pF11β (Cm^R) with SfiI-NotI (Veiga et al., Mol Microbiol 1999, 33 (6): 1232-43), under the control of the pLac promoter, rendering the plasmid pVamyβ (SEQ ID NO: 1). Said plasmid, $pV_{amy}\beta$, was digested with

XbaI-HindIII and the approximately 1.9 kb fragment containing the $V_{amy}\beta$ hybrid was cloned into plasmid pVLT35 (Sp^R) (Lorenzo et al., Gene 1993, 123 (1): 17-24), under the control of pTac promoter, giving the plasmid $pVV_{amy}\beta$.

1.2 Construction of the C-IgAP and $2V_{amy}$ hybrid protein ($2V_{amy}\beta$)

An approximately 0.4 kb DNA fragment encoding V_{amy} was amplified by PCR using the Linker-A48-V_{amy}A (SEQ ID NO: 5) and V_{amy}-Not (SEQ ID NO: 6) primers of the phagemid A10OR3A2 (see 1.1 above). The V_{amy} fragment was bound to the oligonucleotide Linker-A48 (SEQ ID NO: 7), which encodes the scFv A48III-1 flexible peptide (Proba et al., J. Mol. Biol. 1998, 275(2): 245-253), by a PCR reaction without primers, due to the homology between the first 24 bases of the Linker-A48-V_{amy}A and Linker-A48. This reaction was used for the amplification of the Linker-V_{amy} fusion product, using the oligonucleotides V_{amy}-Not (SEQ ID NO: 6) and Linker-A48-V_{amy}-eagl (SEQ ID NO: 8) as primers. The obtained PCR product (Linker-V_{amy}) was digested with NotI and cloned into plasmid $pVV_{amy}\beta$ digested with the same enzyme. The new plasmid $p2V_{amy}\beta$ encodes for a hybrid protein made up of two V_{HH} ($V_{amy}\beta$) bound by a flexible linker and fused to the C-IgAP. The amino acid sequence of said flexible linker is -TPSHNSHQVPSAGGPTANSG- (amino acid one-letter code).

1.3 Construction of the C-IgAP and $3V_{amy}$ hybrid protein ($3V_{amy}\beta$)

The product of the previous PCR reaction (Linker-V_{amy}) was digested with NotI and cloned into $p2V_{amy}\beta$ digested with NotI. The new plasmid, called $p3V_{amy}\beta$, encoded a hybrid protein made up of three V_{HH} ($V_{amy}\beta$) bound by a flexible linker and fused to the C-IgAP. The amino acid sequence of said flexible linker is -TPSHNSHQVPSAGGPTANSG- (amino acid one-letter code).

EXAMPLE 2

Construction of pVLMB10β: V_{LMB10} domain fused to C-IgAP

A hybrid protein anchored to the external surface of the OM of *E. coli* comprising the V_{LMB10} domain fused to the C-IgAp domain, hereinafter referred to as $V_{LMB10}\beta$ (Figure 3B), has been obtained. The signal peptide of PelB drives the secretion of said hybrid protein through the IM to the periplasmic space.

PCR was carried out with oligos VL1 (SEQ ID NO: 9) and VL2 (SEQ ID NO: 10) and using the pCES1-VLMB10 clone (van den Beucken et al., J. Mol. Biol. (2001) 310, 591-601) as template. The amplified 0.47 kb DNA fragment, containing the V_L domain, was digested with XbaI and NotI, and was cloned into plasmid $pV_{amy}\beta$, digested with the same enzymes, replacing the XbaI-NotI fragment which contains the V_{amy} domain in said plasmid. The constructed clone pVLMB10β (SEQ ID NO: 2), which expresses the hybrid protein $V_{LMB10}\beta$ under the control of promoter pLac, was checked by means of DNA sequencing.

EXAMPLE 3

Expression of pVVamyβ in *E. coli* UT5600 cells

The expression of the $V_{amy}\beta$ hybrid protein in *E. coli* UT5600 cells was analyzed in Western-blots using the anti-E-tag mAb (Figure 4). $V_{amy}\beta$ was expressed in a stable manner as a 66 kDa protein, corresponding to the expected size for the fusion (Figure 4A, lane 1). However, the expression of FvHβ (Veiga et al., Mol. Microbiol. 1999, 33(6): 1232-1243), which gave rise not only to the complete 80 kDa protein, but rather to different bands of smaller sizes corresponding to the proteolytic degradation of the hybrid protein (Figure 4A, lane 2). Transformation of *E.coli* cells was carried out by conventional techniques (Ausubel et al., 1994. Current Protocols in Molecular Biology. John Wiley & Sons, New York; Sambrook et al., 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York).

EXAMPLE 4

Exposure on the surface of *E. coli* and folding of the $V_{amy}\beta$ passenger domains

The exposure on the surface of the $V_{amy}\beta$ passenger domains was checked by means of the digestion of *E. coli* cells expressing $V_{amy}\beta$ with trypsin. The samples were analyzed in Western-blot using the anti-E-tag mAb, or a rabbit anti-OmpA serum. In these experiments, the proteolysis of the $V_{amy}\beta$ hybrid proteins in the presence of trypsin (Figure 4B) was checked, which indicates that these passengers were completely exposed on the *E. coli* surface. As previously described, the passenger of the FvHβ fusion was very weakly digested by the action of the trypsin (Figure 4B). The presence of the OmpA protein in all the lanes is shown as an internal loading control in the experiment. The absence of OmpA proteolysis (Figure 4B, lanes 3 and 7) shows the integrity of the OM. When the OM is artificially permeabilized with EDTA, there is an approximately 25 kDa band of OmpA proteolysis (T-OmpA) (Figure 4B, lanes 4 and 8).

In order to know the folding state of these chimeric passenger domains exposed towards the extracellular medium, the specific binding capacity of the Ig domains to their antigen was used. In order to assay binding activity, ELISA plates were covered with recognized antigens of the $V_{amy}\beta$ passenger domain (α-amylase). BSA was used as an antigen control. *E. coli* cells which expressed the C-IgAP hybrids were incubated on ELISA plates and the binding to the Ig passenger was detected by means of the anti-E-tag mAb conjugated with POD. As is shown in Figure 5, the Ig passenger domain $V_{amy}\beta$ exposed towards the extracellular medium exhibited specific binding activity to its antigen, which indicates that it was correctly folded. As a negative control of these experiments, *E. coli* cells expressing the HEβ hybrid protein were used, said hybrid protein consisting of a poly-His segment fused to

the C-terminal β-domain of the *N. gonorrhoeae* IgA protease (Veiga et al., 2002, 21 (9) 2122-31).

EXAMPLE 5

Translocation of the $2V_{amy}\beta$ and $3V_{amy}\beta$ hybrid proteins

$2V_{amy}\beta$ and $3V_{amy}\beta$ hybrid proteins were obtained as it is shown in Figure 7A. By means of proteolysis assays with trypsin, the translocation of the passenger domains ($2V_{amy}$ and $3V_{amy}$) was determined. It was found that the translocation of the $2V_{amy}\beta$ and $3V_{amy}\beta$ passenger domains was complete. In said figure, the DNA constructions encoding the hybrid proteins are shown, the signal peptide is removed during the passage of the protein across the periplasm and, finally, the hybrid proteins do not contain it.

EXAMPLE 6

Expression of pVLMB10β in *E. coli* UT5600 cells

E. coli UT5600 cells were transformed with pVLMB10β and were grown at 30°C in LB-agar plates (1.5% w/v) containing chloramphenicol (40 µg/ml) and glucose (2% w/v). Liquid LB cultures containing chloramphenicol (40 µg/ml) and glucose (2% w/v) were inoculated with these transformed clones, and they were grown at 30°C until the OD_{600} was about 0.5. Then, the cells were harvested by centrifugation (4000xg, 5 min) and were resuspended in liquid LB medium containing chloramphenicol (40 µg/ml) and 0.1 mM IPTG for the induction of the pLac promoter. After 3 hours of incubation at 30°C, the induced cells were harvested by centrifugation and were analyzed by means of proteolysis with trypsin and Western-blot with anti-E-tag-POD.

Figure 6 shows a digestion with trypsin developed with anti-E-tag and OmpA (note that the trypsin lane is number 2). Exposure of over 90% of the $V_{LMB10}\beta$ fusion protein (65 kDa) was

observed. A single, proteolytic degradation band of about 55 kDa, further appeared.

A peptidoglycan (PG) accessibility assay was also carried out, which included the control (pAKnot), $V_{amy}\beta$, $2V_{amy}\beta$, $3V_{amy}\beta$ and V_{LMB10} constructs. In all of them, only a PG accessibility level appeared at about 5% maximum and identical to that observed in the control strain (see Figure 8) without expression of any hybrid protein.

EXAMPLE 7

Assay for specific adhesion to an antigen

In order to study the specific adhesion capacity of *E. coli* cells which expressed the hybrid proteins (comprising C-IgAP) in ELISA plates coated with recognized antigens of the passenger domain of the hybrid protein, *E. coli* UT5600 cells expressing the $V_{amy}\beta$ hybrid protein were incubated in ELISA plates which had previously been coated with α-amylase antigen or control antigen (BSA) and the binding to the cells with a polyclonal anti-*E. coli* antibody generated in mice was revealed, and the binding of these antibodies with a mouse anti-IgGs antibody conjugated to peroxidase was detected (Figure 9). As a negative control, *E. coli* cells expressing the HEβ hybrid protein were used. The results obtained show the specific adhesion of bacteria having on the external surface of its OM a single-domain recombinant antibody (V_{amy}) in the form of a hybrid protein ($V_{amy}\beta$) to its antigen (α-amylase). Therefore, the hybrid protein $V_{amy}\beta$ directs the adhesion of said bacteria depending on the antigen recognized by the Ig domain.